Cellular Immunology 271 (2011) 308–318

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Synthetic adjuvants for vaccine formulations: Evaluation of new phytol derivatives in induction and persistence of specific immune response

Youssef Aachoui [a], Michael L. Schulte [b], Richard W. Fitch [b], Swapan K. Ghosh [a,*]

[a] Department of Biology, Indiana State University, Terre Haute, IN 47809, United States
[b] Department of Chemistry, Indiana State University, Terre Haute, IN 47809, United States

ARTICLE INFO

Article history:
Received 25 March 2011
Accepted 14 July 2011
Available online 29 July 2011

Keywords:
Diterpene adjuvants
Adjuvanticity of phytol derivatives
T-helper polarization
Role of apoptosis/necrosis

ABSTRACT

Terpenoids are ubiquitous natural compounds that have been shown to improve vaccine efficacy as adjuvants. To gain an understanding of the structural features important for adjuvanticity, we studied compounds derived from a diterpene phytol and assessed their efficacy. In a previous report, we showed that phytol and one of its derivatives, PHIS-01 (a phytol-derived immunostimulant, phytanol), are excellent adjuvants. To determine the effects of varying the polar terminus of PHIS-01, we designed amine and mannose-terminated phytol derivatives (PHIS-02 and PHIS-03, respectively). We studied their relative efficacy as emulsions with soluble proteins, ovalbumin, and a hapten-protein conjugate phthalate-KLH. Immunological parameters evaluated consisted of specific antibody responses in terms of titers, specificities and isotype profiles, T cell involvement and cytokine production. Our results indicate that these new isoprenoids were safe adjuvants with the ability to significantly augment immunogen-specific IgG1 and IgG2a antibody responses. Moreover, there was no adverse phthalate cross-reactive anti-DNA response. Interestingly, PHIS-01 and PHIS-03 influenced differentially T-helper polarization. We also observed that these compounds modulated the immune response through apoptotic/necrotic effects on target tumor cells using murine lymphomas. Finally, unlike squalene and several other terpenoids reported to date, these phytol derivatives did not appear arthritogenic in murine models.

1. Introduction

Vaccine efficacy depends largely on two variables; the specific antigen(s) used and the choice of adjuvants. It is the inclusion of the latter in a vaccine formulation that significantly improves the quality and magnitude of specific immune response. Adjuvants are a diverse group of chemical compounds and vary widely in their ability to influence immune response. Selection of adjuvants is generally empirical, and the list of experimental adjuvants is growing. However, a major concern with experimental adjuvants is their potential for acute or chronic toxicity. Only a few adjuvants, including hydrophilic aluminum salts (Alum) [1] and a hydrophobic squalene-based emulsion (MF59) are licensed for human use [2,3]. For veterinary purposes, oil-in-water emulsions such as Freund's adjuvants (FA, paraffin oil with or without mycobacterial components) and TiterMax/Ribi's adjuvants (containing squalene) have been used [4,5].

Several studies with oil-in-water emulsion adjuvants have shown that they help retain immunogens longer, an important parameter for good adjuvanticity. In addition, they promote activation and maturation of antigen presenting cells [6–8]. It has also been suggested that they may induce danger signals to alert the immune systems against a potential threat, but without much adverse inflammatory response [9]. Since no single adjuvant has been shown effective in every situation, there is an ever-growing need for new adjuvants. Ideally, an adjuvant should have little reactogenicity, but be broadly effective in modulating the host-immune microenvironment. However, a central issue with adjuvants is empiricism in their selection, as mentioned earlier. This difficulty is largely due to a lack of any systematic, correlative structure–function studies.

The physicochemical properties necessary for immunomodulation by oil-in-water adjuvants are not fully understood. These emulsions function in many capacities from membrane anchoring

Abbreviations: PHIS, phytol-based immune-stimulant; KLH, keyhole limpet hemocyanin; β-CyD, β-cyclodexterin; DMEM, Dulbecco's modified Eagle's minimal essential medium; ELISA, enzyme linked immunosorbant assay; APC, antigen-presenting cell; DMSO, dimethyl sulfoxide; PI, propodium iodide; LPS, lipopolysaccharide; CTL, cytotoxic T lymphocyte; CDC, complement dependent cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; OPD, *o*-phenylene diamine; HRP, horseradish peroxidase; PBS, phosphate buffer saline.

* Corresponding author. Fax: +1 812 237 3378.
E-mail addresses: yaachoui@indstate.edu (Y. Aachoui), michael.l.schulte@vanderbilt.edu (M.L. Schulte), richard.fitch@indstate.edu (R.W. Fitch), sghosh@indstate.edu (S.K. Ghosh).

0008-8749/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellimm.2011.07.009

to cell signaling [10,11]. Many natural isoprenoids including vitamin E and squalene are known for their beneficial effects on the immune system. However, squalene (a natural triterpene adjuvant from shark liver oil) has been shown to have adverse effects in rodents [12,13]. Furthermore, adverse effects have been reported with the naturally occurring diterpene pristane. Though an effective adjuvant, pristane has proven to be an inducer of rodent arthritis and plasmacytomas [12,14,15]. Similarly, phytol a natural diterpene alcohol in chlorophyll, although an effective adjuvant, produced adverse effects including splenomegaly, hepatotoxicity, and tumor promotion in rodents [16–18].

To ascertain if these problems could be overcome, we have developed a series of phytol-based immunostimulants including PHIS-01, PHIS-02, and PHIS-03 by chemical modifications of phytol (US patent pending 11/295131). In previous reports, we established that PHIS-01 (phytanol) is an effective adjuvant [16,19,20]. It is stable and has no detectable toxicity. It can enhance both humoral and cell-mediated immunity, and can exert ameliorating effects in lupus-prone NZB/WF1 mice. The efficacy of PHIS-01 led us to explore the importance of its polar alcoholic group, PHIS-02 (phytanyl amine) was produced from PHIS-01 by conversion to the bromide and Gabriel synthesis to give the amine [21,22]. PHIS-03 was prepared by mannosylation of PHIS-01 with pentaacetylmannose using the trichloroacetimidate method [21–23]. In this study, we compared PHIS-02 and PHIS-03 with PHIS-01 and other commonly used adjuvants and determined to what extent their adjuvanticity depends on apoptotic/necrotic processes for activation of antigen-presenting cells, and subsequently the acquired immune response. Using two soluble, potentially autoimmunogenic proteins, ovalbumin and a hapten-protein conjugate phthalate-keyhole limpet hemocyanin (KLH), we assessed titer, specificity, and isotypic profiles of antibody response as well as T cell proliferation and cytokine production. We report that modified phytol-derived adjuvants significantly augment antibody response of isotypes IgG1 and IgG2a, promote effective T cell proliferation and exhibit no adverse autoimmune anti-DNA response. We also noted that these phytol derivatives function by activation of antigen-presenting cells involving apoptotic/necrotic effects on target cells. In the accompanying paper, we determined how apoptotic/necrotic effects influence expression profile of inflammation-related cytokine and chemokine genes.

2. Materials and methods

2.1. Animals

C57Bl/6 and BALB/c mice are in-bred strains with known genetic backgrounds; they are widely used in vaccine research as models of human diseases [14–16,24,25]. For this study, we used female BALB/c and C57Bl/6 mice (6–8 weeks of age). They were housed in the animal facility of Indiana State University according to principles of laboratory animal care (NIH publication 85–23) followed under a specific protocol approved by the Institutional Animal Care and Use Committee (ACUC) of Indiana State University.

2.2. Chemicals

Reagents used in this study were from the following sources: ortho-phthalate (Pfaltz and Bauer Inc., Waterbury, CT); β-cyclodextrin, calf thymus DNA, rabbit anti-mouse immunoglobulin-horseradish peroxidase (Ig-HRP) reagent, *o*-phenylene diamine (OPD), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Alum, squalene, Annexin V apoptosis kit, methylated bovine serum albumin (mBSA) and BSA (Sigma Chemical Co., St. Louis, MO); KLH (Calbiochem, CA); Dulbecco's modified Eagle's minimal essential medium (DMEM), isotyping kit (Invitrogen., Carlsbad, CA); polyvinyl 96-well flat bottom plates (Falcon). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay kit, Promega, Madison, WI), cytoTox96 non-radioactive cytotoxicity kit and Wizard® SV Genomic DNA Purification System kit (Promega, Madison, WI). The phytol derivatives, PHIS-02 (phytanyl amine) and PHIS-03 (phytanol mannose) (US patent pending 11/295131) were obtained by chemical modification of phytanol as described [21–23,26]. All reagents and chemicals used for the synthesis were ACS grade, and all new compounds gave satisfactory NMR, IR, and MS data.

2.3. Preparation of vaccine formulation

Ortho-phthalate-protein conjugates were prepared by azo-coupling the diazotized 4-aminophthalic acid (disodium) to KLH as described by Ghosh et al. [27]. Briefly, 200 μL of phthalate-KLH (100 μg/mice) was emulsified in equal volumes of either PHIS-01 (40 mg) PHIS-02 (2.5 mg), PHIS-03 (5 mg), complete or incomplete Freund's adjuvants (CFA) or (IFA), squalene, or adsorbed on alum by vigorously mixing a few times with a syringe and vortex. The emulsion prepared was given intraperitoneally in a volume of 400 μL to 6 to 8-week old mice (six mice per group). Mice are given two booster injections at 10 day-interval, and bled 5 days after each immunization through retro-orbital veins. The parallel control groups of mice were immunized with ortho-phthalate-KLH in PBS.

2.4. Assessment of serum levels of anti-phthalate and anti-DNA antibodies

Determination of levels of anti-phthalate and anti-DNA antibodies was assessed by enzyme linked immunosorbent assays (ELISA). ELISA plates were coated for 2 h at 37 °C with 50 μL of 10 μg/mL of either phthalate conjugated to BSA or calf thymus DNA at 4 °C. The plates were washed four times with PBS containing 0.01% Triton X-100, blocked overnight with 1% BSA and washed again. Serial dilutions (10–10,000-fold) of test sera from normal and immunized mice were added in triplicate to the plates, and incubated at 37 °C for 1 h. Following incubation and after washing four times with PBS/Triton X-100, rabbit anti-mouse immunoglobulin-horse-radish peroxidase (HRP) (50 μL) (at 1:3000 dilutions) was added. Plates were incubated for 1 h and washed again. The rabbit anti-mouse immunoglobulin-HRP was detected by addition of *o*-phenyl diamine (OPD). The reaction was stopped by adding 50 μL of 10% H_2SO_4, and the intensity of color was determined at OD 490 nm.

In separate experiments, ELISA plate were coated with ortho-phthalate structural analogs like meta-phthalate and para-phthalate, in order to test specificity of antibody produced toward ortho-phthalate as described previously [28,29].

2.5. Antibody isotypes

To determine isotypes of anti-phthalate specific antibodies produced, sera from different groups of immunized mice (second immunization), were diluted 1:100, and tested in triplicate on phthalate-coated plates, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

2.6. Detection of cytokines by sandwich ELISA

Determination of cytokine levels in supernatant taken from T-cell proliferation was done by sandwich ELISA, following the protocol provided by eBioscience (IL-4, INF γ, and IL-2 ELISA Kits). ELISA plates were coated with 100 μL/well of capture antibody and incubated overnight at 4 °C. After washing the plates five times with